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Antigen Crosspresentation by Human Plasmacytoid Dendritic Cells

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SUMMARY

Crosspresentation is a specialized function of myeloid dendritic cells (mDCs), allowing them to induce CD8$^+$ T cell responses against exogenous antigens that are not directly produced in their cytotosol. Human plasmacytoid DCs (pDCs) are not considered so far as able to perform crosspresentation. We showed here that purified human pDCs crosspresented vaccinal lipopeptides and HIV-1 antigens from apoptotic cells to specific CD8$^+$ T lymphocytes. Apoptotic debris were internalized by phagocytosis and the lipopeptide LPPol reached nonacidic endosomes. This crosspresentation was amplified upon influenza virus infection. Importantly, the efficiency of crosspresentation by pDCs was comparable to that of mDCs. This property of human pDCs needs to be taken into account to understand the pathogenesis of infectious, allergic, or autimmune diseases and to help achieve desired responses during vaccination by targeting specifically either type of DCs.

INTRODUCTION

Plasmacytoid dendritic cells (pDCs) are a subset of DCs present in the blood and in lymphoid organs (Cella et al., 2000; Grouard et al., 1997). Their main function is the production of type I interferons (IFN) in response to microbial stimuli (Cella et al., 2000; Ito et al., 2006; Kadowaki et al., 2000; Siegal et al., 1999). pDCs die rapidly by apoptosis unless rescued by IL-3, type I IFN, or factors inducing type I IFN secretion (Grouard et al., 1997). They express CD4 and major histocompatibility complex (MHC) class II molecules but lack molecules specific for the T, B, NK, or monocytic lineages. pDCs also express BDCA-2, a specific membrane lectin (Dzionek et al., 2001), CD123, the α chain of the IL-3 receptor, and neuropilin-1 (BDCA-4).

DCs are the only antigen-presenting cells (APCs) that stimulate naive T lymphocytes (Banchereau and Steinman, 1998). CD8$^+$ T lymphocytes are essential in immune responses to viruses and tumors. They recognize peptide-class I MHC complexes. These complexes are generated either after direct presentation, as a result of endogenous production of the antigens in the cytosol, or after crosspresentation, which occurs after internalization of exogenous antigens into endocytic compartments, followed in most of the cases by export into the cytosol. This allows presentation of antigens from microorganisms that do not infect DCs, from tumor cells, and from autologous cells (Bevan, 1976). In the cytosol, antigens are generally digested by the proteasome (Seifert et al., 2003), further trimmed by other enzymes, transported into the endoplasmic reticulum, and then associated with the heavy chain of MHC class I molecules and with β2-microglobulin, allowing export to the cell surface and presentation to CD8$^+$ T lymphocytes (Cresswell et al., 2005; Rock and Shen, 2005). Crosspresentation may be the predominant antigen-presentation pathway for many infections, depending on the availability of DCs at the site of infection and on their infectability (Chen et al., 2004; Heath et al., 2004; Rock and Shen, 2005). Particularly, viral proteins from apoptotic, infected cells can be crosspresented by DCs (Albert, 2004).

T cell responses to antigen presentation depend on three signals. Signal 1 is the antigen-specific signal mediated by the MHC-peptide complex. Signal 2 is mediated by interactions of CD40, CD80, CD86, and other costimulatory molecules with their T cell ligands. Signal 3 is the polarizing signal, mediated by soluble or membrane-bound

factors, such as interleukin-12 (IL-12), type I IFN, or IL-10. Signal 3 promotes the development of either T helper type 1 (Th1), T helper type 2 (Th2), or regulatory T (Treg) cell responses (Kapsenberg, 2003). Sensitization of naive T lymphocytes by DCs results in immunity or tolerance, depending on modulation of the three signals by the environment and on the nature of the DCs (Albert et al., 2001; Moser, 2003; O'Garra and Vieira, 2004; Reis e Sousa, 2006; Shortman and Heath, 2001; Sporri and Reis e Sousa, 2005; Steinman et al., 2003). Toll-like receptor (TLR) ligands like imiquimod (R-837) and CpG oligonucleotides are recognized by TLR 7 and 9, respectively, on pDCs, whereas bacterial wall-derived LPS is recognized by TLR 4 on mDCs. Binding of these ligands induces maturation of the respective DCs, including enhancement of the three signals governing T cell stimulation. Combination of intracellular and extracellular TLR signaling leads to synergic responses (Napolitani et al., 2005; Trinchieri and Sher, 2007).

Antigen presentation by DCs has mostly been studied in human monocyte-derived DCs and in murine bone-marrow-derived or spleen DCs (Guermonprez et al., 2002). pDCs were first considered to have a minor role in antigen uptake and presentation (Grouard et al., 1997). Nevertheless, human blood pDCs internalize Lucifer yellow (Dzionek et al., 2001) and antibody-bound MHC class II-restricted antigens (Benitez-Ribas et al., 2006; Dzionek et al., 2001; Zhang et al., 2006), and mouse spleen pDCs phagocytose latex beads (Ochando et al., 2006).

The role of pDCs in T cell polarization has been mainly studied in mixed lymphocyte reactions. After stimulation by influenza A virus (IAV) or by CD40 ligand (CD40L), pDCs are as efficient as mDCs to induce strong type 1 T helper cell proliferation and high production of IFN-γ (Cella et al., 2000). After stimulation with IL-3 or herpes simplex virus (HSV)-1, they induce naive $CD4^+$ T cells to produce IL-4 or IL-10, respectively (Kadowaki et al., 2000). In addition, human pDCs can induce Treg cells in vitro (Gilliet and Liu, 2002; Kawamura et al., 2006; Moseman et al., 2004). Human pDCs present peptides in association with MHC class I antigen molecules as effectively as mDCs (Fonteneau et al., 2003; Lore et al., 2003; Salio et al., 2003). After exposure to IAV in vitro, pDCs efficiently activate influenza-specific Th1 $CD4^+$ and $CD8^+$ CTL memory responses (Fonteneau et al., 2003). In vivo, murine pDCs loaded with a lymphocytic choriomeningitis virus peptide induce primary $CD8^+$ T cell responses when they are preactivated by IAV (Schlecht et al., 2004). Therefore, pDCs perform most of the functions that are necessary for crosspresentation, i.e., endocytosis, processing, and presentation of viral antigens, and mature pDCs display full T cell stimulation capacity. However, murine pDCs were claimed to be unable to induce crosspriming in vivo (Salio et al., 2004). In addition, in contrast to mDCs, human pDCs do not crosspresent the full-length tumor protein NY-ESO-1 in vitro, even when stimulated by TLR ligands or by CD40L (Schnurr et al., 2005). Therefore, it is important to assess whether pDCs perform crosspresentation in a physiological manner.

We tested crosspresentation of a candidate HIV lipopeptide vaccine that is endocytosed and crosspresented by monocyte-derived DCs to $CD8^+$ T lymphocytes specific for HIV-1 epitopes (Andrieu et al., 2003). We also used, as a source of antigens, apoptotic cells expressing HIV proteins. Here we demonstrate that human blood pDCs were able to perform crosspresentation.

RESULTS

Purified Human pDCs Crosspresent Vaccinal Antigens to Specific T Lymphocytes

Lipopeptides containing peptides from several proteins from the Lai sequence of HIV-1 ($HIV\text{-}1_{Lai}$), coupled to the tetanus toxoid $TT_{830\text{-}843}$ helper peptide, are currently used in clinical trials in volunteers and HIV-infected patients (Durier et al., 2006). Lipopeptides are crosspresented by monocyte-derived DCs and induce $CD4^+$ and $CD8^+$ T cell responses (Andrieu et al., 2003; Gahery et al., 2005). To test crosspresentation by pDCs, BDCA-4^+ cells were selected positively after depletion of other cells from peripheral blood mononuclear cells (PBMCs) obtained from healthy donors (Figure 1A). Purified pDCs were incubated with a vaccinal lipopeptide preparation, and IAV was added to induce pDC maturation. Plasmacytoid DCs were then washed and cultured with a $Gag_{77\text{-}85}$-specific primary T cell line. An efficient antigen-specific T cell stimulation by pDCs was evidenced by IFN-γ ELISPOT (Figure 1B). Similar results were obtained with two single lipopeptides including either the $HIV\text{-}1_{Lai}$ $Nef_{66\text{-}97}$ (LPNef) or $Pol_{461\text{-}484}$ sequence (LPPol) (Andrieu et al., 2003) and specific T cell lines (Figure 1B). MHC class I restriction was assessed by immunomagnetic depletion of the $CD8^+$ (81%) or the $CD4^+$ (14%) T cells contained in the $Pol_{476\text{-}484}$-specific T cell line: IFN-γ responses required $CD8^+$ and not $CD4^+$ T cells (Figure 1C). These data show that pDCs crosspresent different vaccinal lipopeptides to $CD8^+$ T cells specific for different epitopes and restricted by different MHC class I antigens.

Plasmacytoid DCs were then compared to conventional mDCs for crosspresentation of the LPPol lipopeptide. Purified pDCs or mDCs were incubated for 12 hr with LPPol, then washed and cultured with a $Pol_{476\text{-}484}$-specific T cell line. Plasmacytoid DCs and mDCs induced similar IFN-γ responses to the lipopeptide (Figure 2A). Lipopeptide and epitopic peptide presentation were increased by incubating mDCs with LPS or pDCs with IAV; conversely, LPS did not enhance presentation by pDCs, which lack TLR 4, and IAV did not enhance presentation by mDCs (Figure 2A), perhaps because it was cytotoxic for those cells, as noted before (Cella et al., 2000; Fonteneau et al., 2003). Purified pDC populations may contain a few contaminating mDCs. To test whether small numbers of mDCs would stimulate T cell responses as efficiently as the whole pDC population, experiments were performed with decreasing numbers of pDCs or mDCs. T cell responses decreased proportionally to the number of mDCs and were undetectable at numbers higher than those potentially corresponding to the few contaminating

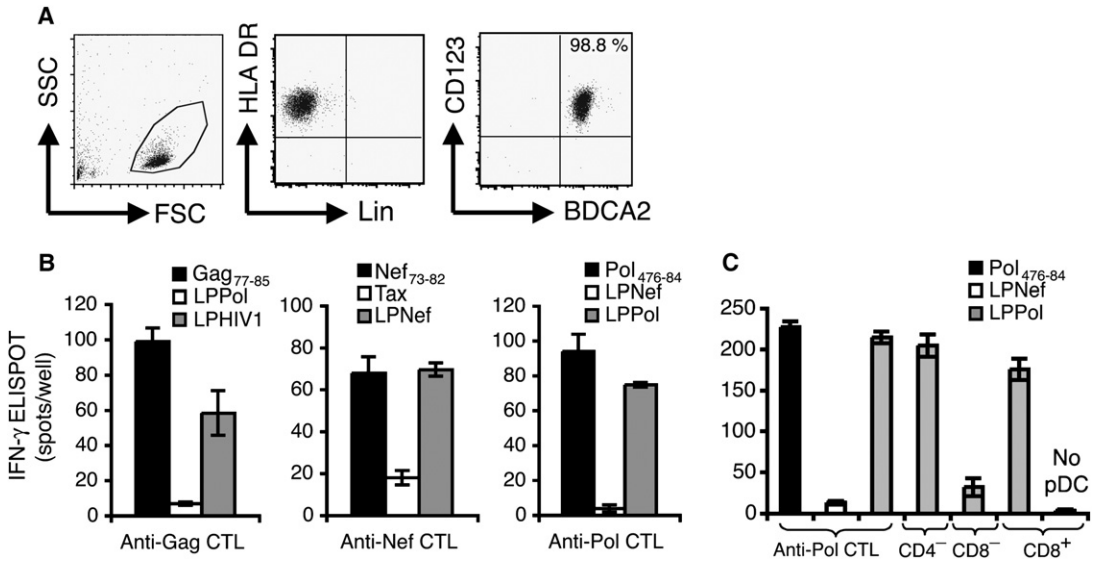

Figure 1. Plasmacytoid DCs Crosspresent Antigens from Vaccinal Lipopeptides

(A) pDCs were purified with BDCA4 antibody-coated magnetic beads from PBMCs after depletion of non-pDC cells. Purity was assessed by FACS by forward and side scatter characteristics (FSC and SSC), expression of HLA-DR but not of different cell lineage (Lin)-specific molecules, and expression of the IL3 receptor α chain (CD123) and of BDCA2, a pDC-specific lectin.

(B) Left: pDCs were loaded with the vaccinal lipopeptide preparation LPHIV1, then cultured in the presence of influenza A virus; they were used as APCs to stimulate HIV-1 Gag_{77-85}-specific, HLA-A2-restricted, primary $CD8^+$ T cells in an IFN-γ ELISPOT assay. Middle: lipopeptide LPNef-loaded pDCs, cultured in the presence of CpG, were used to stimulate Nef_{73-82}-specific, HLA-A3-restricted T cells. Right: lipopeptide LPPol-loaded pDCs, cultured in the presence of R-837, were used to stimulate $Pol_{476-484}$-specific, HLA-A2-restricted T cells. Tax, HTLV-1 HLA-A2-restricted peptide.

(C) $CD8^+$ T cell dependence of the response. pDCs were incubated with either control peptides or LPPol, then with the unseparated $Pol_{476-484}$-specific T cell line, or with different cell fractions. Alternatively, the T cell line was incubated with LPPol and no DCs. Data are represented as mean ± SEM (n = 3 replicates) and representative of at least two independent experiments.

mDCs in pDC populations (Figure 2B). This was also true when responses were stimulated by LPS (that increased responses induced by mDCs) or IAV (that increased responses induced by pDCs) (Figure 2B). B lymphocytes, which are also APCs, do not account for the presentation activity found in the pDC population (Figure S1 in the Supplemental Data available online). If small numbers of mDCs by themselves cannot account for antigen presentation, they might have been stimulated when mixed with pDCs. Indeed, in mixed populations, it was reported that HIV induces IFN-α secretion by pDCs, which in turn induce bystander mDC maturation (Fonteneau et al., 2004). However, adding 10% or 20% of contaminating mDCs to purified pDCs did not change T cell responses, showing that mDCs did not synergize with pDCs for crosspresentation (Figure 2C). Therefore, pDCs crosspresent the $Pol_{476-484}$ epitope from the lipopeptide to specific T lymphocytes, and this presentation is not attributable to contaminating APCs.

Lipopeptide Uptake and Crosspresentation by pDCs

We then assessed by flow cytometry and confocal microscopy how pDCs take up the Pol lipopeptide. The Rhodamine-labeled lipopeptide analog (LPPol-Rho) (Andrieu et al., 2003) was first incubated with fresh PBMCs (Figure S2). Even as a minor population of 0.83% among PBMCs, pDCs efficiently captured the lipopeptide like mDCs. Purified pDCs also internalized the lipopeptide rapidly, through an active, temperature-dependent mechanism (Figure 3A). After 1 hr, pDCs were homogenously labeled in intracellular compartments reminiscent of endosomal structures. To assess whether the lipopeptide was internalized through endocytosis, purified pDCs were first surface-labeled with BDCA-2 mAbs at 4°C, then washed and incubated with lipopeptide at 37°C, allowing simultaneous endocytosis of BDCA-2 bound antibodies and of the lipopeptide. After 30 min, the lipopeptide colocalized with BDCA-2-labeled endosomes in most of the pDCs (Figure 3B). Single channel intensity false-color labeling revealed a clear localization of BDCA-2 in endosomes, whereas the lipopeptide appeared in addition in a larger, fuzzy zone around endosomes (Figure 3B, middle). Recent reports suggest that efficient MHC class II-restricted antigen presentation correlates with the induction of signaling events from endosomes containing both the antigen and a TLR ligand (Blander and Medzhitov, 2006). We investigated whether the lipopeptide used the same compartment as the TLR-9 ligand CpG B-FITC, complexed to DOTAP so as to gain access to early endosomes like CpG A (Guiducci et al., 2006; Honda et al., 2005). The lipopeptide was found underneath the surface of BDCA-2-labeled extensions and colocalized with CpG B-DOTAP in intracellular

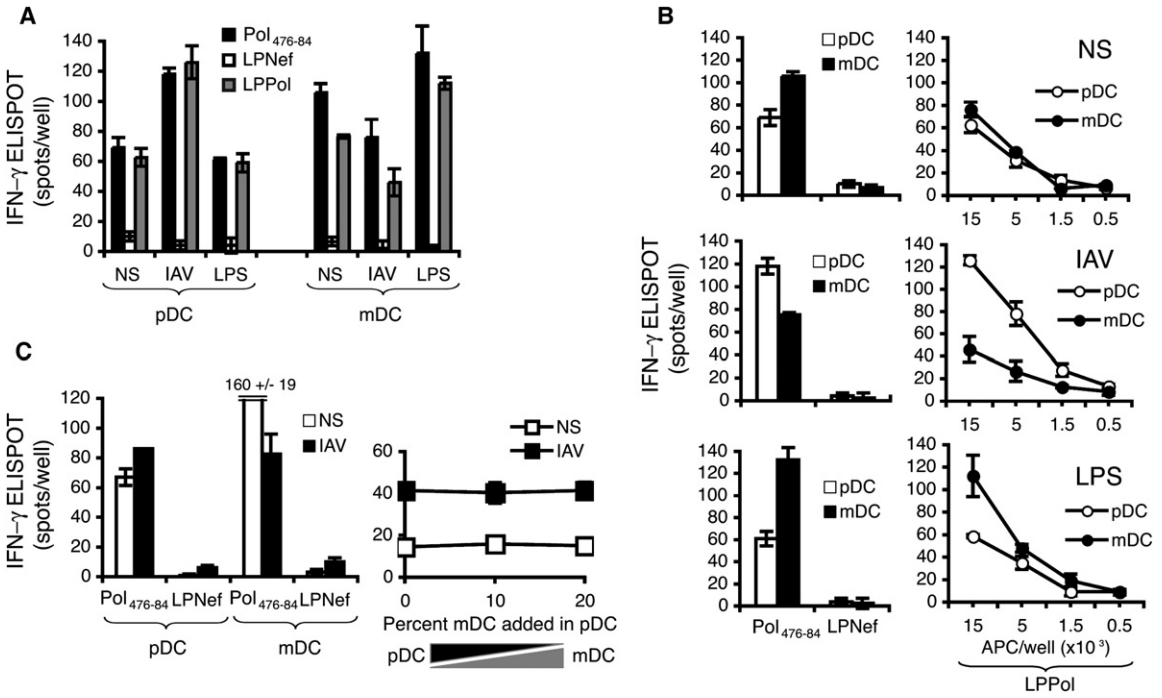

Figure 2. Crosspresentation by pDCs, Not Contaminating mDCs

(A) Crosspresentation by purified pDCs or mDCs. Plasmacytoid DCs and mDCs were loaded with LPPol, the negative control LPNef, or the positive epitope control $Pol_{476-484}$, and left unstimulated (NS) or stimulated with either IAV or LPS; they were then incubated with a $CD8^+$ T cell line specific for $Pol_{476-484}$. Results are shown here for 15,000 DCs and 45,000 T cells/well, as the number of IFN-γ ELISPOTS/well.

(B) Small numbers of mDCs do not achieve crosspresentation. Left, control responses to $Pol_{476-484}$- or LPNef-loaded DCs; right, $CD8^+$ T cells response to decreasing numbers of LPPol-loaded DCs unstimulated (top) or stimulated with IAV (middle) or LPS (bottom).

(C) No synergistic effect between pDCs and mDCs for antigen crosspresentation. Left: control responses to purified pDCs or mDCs as in (B). Right: different proportions of mDCs were added to pDCs before loading with LPPol. The final number of APCs is equal to 10,000/well in the IFN-γ ELISPOT assay. Data are represented as mean ± SEM (n = 3 replicates) and representative of at least two independent experiments.

vesicles (Figure 3C). We then assessed whether the lipopeptide reached acidic, late endosomes or lysosomes, labeled by LysoTracker (Trombetta et al., 2003). Vesicles containing LPPol (Figure 3D, left) were often in close contact with LysoTracker-positive vesicles, without clear-cut colocalization (Figure 3D, middle). If a minor part of the lipopeptide colocalized with LysoTracker, this was in a small proportion of cells and after at least 60 min of incubation (Figure 3D, right). The mean intensity of lipopeptide labeling in the LysoTracker-positive vesicles was lower than in the others. This suggests either that the lipopeptide is degraded in these acidic vesicles or that it moves toward the cytosol before or when it reaches them. These results show that the Pol lipopeptide is internalized into endosomal vesicles where it colocalizes with CpG B-DOTAP, mostly in nonacidic endosomes.

Presentation of LPPol was dependent on intracellular processing by the proteasome, as assessed by dose-dependent inhibition by lactacystin or epoxomycin, which had no or limited toxic inhibitory activity on presentation of the Pol epitope added exogenously (Figures 3E and 3F). Together with the data showing internalization into endosomal compartments, these data confirm the crosspresentation of the lipopeptide.

Purified Human pDCs Internalize and Crosspresent Antigens from Apoptotic Cells Expressing HIV Proteins

Epitopes from apoptotic, infected cells are a major source of antigens for crosspresentation (Albert, 2004; Chen et al., 2004; Heath et al., 2004). HIV-1 antigens originating from apoptotic, infected $CD4^+$ T cells are very efficiently crosspresented by monocyte-derived DCs and mDCs (Marañón et al., 2004). We assessed whether pDCs would also crosspresent antigens from apoptotic, infected cells. Purified pDCs were cultured with PKH-labeled, apoptotic H9 cells that were chronically infected with HIV. Flow cytometry showed that PKH67 was associated with pDCs in a temperature-dependent manner. This association was mostly dependent on actin polymerization, as indicated by the fact that it was inhibited by 80% by latrunculin B and by 60% by cytochalasin D (Figure 4A). Further demonstration of real internalization by phagocytosis was brought by confocal microscopy showing apoptotic fragments in endosome-like structures (Figures 4B and 4C). To assess functional crosspresentation, purified pDCs were cultured with apoptotic H9HIV cells in the presence of 3′-azido-3′-deoxythymidine (AZT) to inhibit viral replication, then washed and used as APCs.

Stimulation of T cell lines specific for different HIV epitopes was evidenced by IFN-γ ELISPOT (Figure 4D). pDCs also crosspresented HIV antigens from apoptotic, infected PBMCs (PHA blasts) to a Gag_{77-85}-specific T cell line (Figure 4E, bottom right). Moreover, PBMCs from an HIV-infected patient underwent strong HIV-specific proliferation after culture with apoptotic, infected PHA blast-loaded pDCs, and not with uninfected PHA blast-loaded pDCs or with infected PHA blasts alone (Figure 4E). Because the antigen donor cells were infected with HIV, pDCs might have been productively infected by HIV and have presented directly antigens derived from endogenous viral proteins. To avoid viral replication, experiments were performed in the presence of antiretroviral drugs (azidothymidine [AZT], a nucleosidic reverse transcriptase inhibitor, or AMD3100[AMD], a fusion inhibitor analog to CXCR4), or H9HIV cells were treated by saquinavir (SQ), a HIV protease inhibitor. Addition of these drugs at concentrations that inhibited HIV-1_{Lai} replication (data not shown) did not decrease T cell stimulation by pDCs incubated with apoptotic H9HIV cells (Figure 5A). Virus production was not detected upon exposure of pDCs with cell-free virus, added at amounts similar to those contained in H9HIV cells or in PHA blasts (Figure S3). Gag-specific IFN-γ responses were not elicited either, and 300 to 1000 times more virus was necessary to induce a relatively low response (Figure 5B).

In addition, we showed crosspresentation with three different HIV-1_{Lai} antigen-donor cells that do not produce viral particles: Jurkat cells transduced with a lentiviral vector vector coding for Nef, CEM clones stably expressing Nef, or fibroblasts transiently transfected with a gag gene. When these cells were irradiated to induce apoptosis and cocultured with pDCs, the pDCs activated the relevant CTLs (Figure 5C). There was a possibility that the donor cells rather than the DCs were directly presenting the antigen. However, the donor cells did not express the HLA molecule that restricted the response of the cell lines, except for the *nef*-transduced Jurkat cells (HLA-A3, which binds HIV-1_{Lai} Nef_{73-82}; Table S1). Presentation and stimulation by the antigen donor cells was ruled out in all the cases, by testing these cells alone without DCs (Figures 4D, 4E, 5C, and 6A). These data show that T cell responses were due to crosspresentation and not to direct viral presentation.

Plasmacytoid DCs were compared to mDCs for crosspresentation of HIV antigens derived from three different apoptotic antigen donor cells: H9HIV cells, infected PHA blasts, or 8E5 cells. 8E5 cells are CEM derivatives containing a defective proviral HIV genome that produces no infectious virus. IFN-γ production increased as a function of the number of either pDC or mDCs in superimposable curves, but not of apoptotic cells alone (Figure 6A). Activation of T cells was blocked with a MHC class I specific antibody in a dose-dependent way (Figure 6B), showing that T cells were activated through TCR and not by pDC-secreted cytokines. Moreover, specific inhibition by epoxomycin underlines the necessity for intracellular processing (Figure 6C). Therefore, human pDCs internalize apoptotic debris from cells expressing HIV antigens and crosspresent them to specific $CD8^+$ T cells.

DISCUSSION

Here we show that human pDCs internalize vaccinal lipopeptides, as well as apoptotic debris from cells expressing HIV antigens, and crosspresent viral antigens to HIV-specific $CD8^+$ T cells, in comparable efficiency to mDCs. pDCs are susceptible to HIV infection (Lore et al., 2005; Patterson et al., 2001), but experimental systems with uninfected, transfected antigen donor cells excluded direct presentation. Here pDCs took up apoptotic cell fragments by phagocytosis. pDCs capture free HIV (Beignon et al., 2005) and microvesicles from VSV-infected cells (Pichlmair et al., 2007). They may also take up apoptotic debris in vivo in different diseases, like systemic lupus erythematosus (Bave et al., 2003), through CD32 (Means et al., 2005) or other pathogen recognition receptors (Blasius and Colonna, 2006). Therefore, crosspresentation by pDCs is likely to play a role in the pathogenesis of HIV infection and of other diseases.

The mechanisms leading to crosspresentation in pDCs is probably similar to those operating in mDCs (Chen et al., 2004; Cresswell et al., 2005; Guermonprez et al., 2002; Rock and Shen, 2005). In human and murine mDCs, crosspresentation occurs after transport of the antigen from the endosomes to the cytosol and digestion by the proteasome (Guermonprez et al., 2002). Endosomal acidification is regulated in immature mDCs (Trombetta et al., 2003): the NADPH oxidase NOX2 maintains alkalinization of early phagosomes, allowing crosspresentation of exogenous ovalbumin instead of degradation (Savina et al., 2006). The lipopeptide used here as an antigenic model for crosspresentation was previously shown to be endocytosed by human mDCs, then digested by the proteasome (Andrieu et al., 2003). In human pDCs, exit of the lipopeptide into the cytosol is suggested by our data showing the diffusion of Rhodamine outside of endosomal compartments. This was reinforced by the requirement for processing by the proteasome, which is localized outside of the endosomes (Guermonprez et al., 2003). Our results showing lower lipopeptide labeling in acidic than in non-acidic vesicles suggest that most of the lipopeptide exits to the cytosol before full lysosomal acidification, although part of the lipopeptide may also undergo degradation within acidic vesicles. The endosomal pathway used by the lipopeptide intersected with that of CpG, which stimulated crosspresentation in our experiments. Therefore, crosspresentation of the lipopeptide in pDCs is dependent on an endosome-to-cytosol pathway and may depend on regulated acidification of the endosomal compartments, similar to crosspresentation in mDCs (Savina et al., 2006).

We showed here that crosspresentation by pDCs resulted in the activation of effector T cell responses such as IFN-γ secretion and cell proliferation. This activation probably does not require type I IFN production, because it was observed in unstimulated or IL-3-treated pDCs. However, it was strongly stimulated by influenza virus.

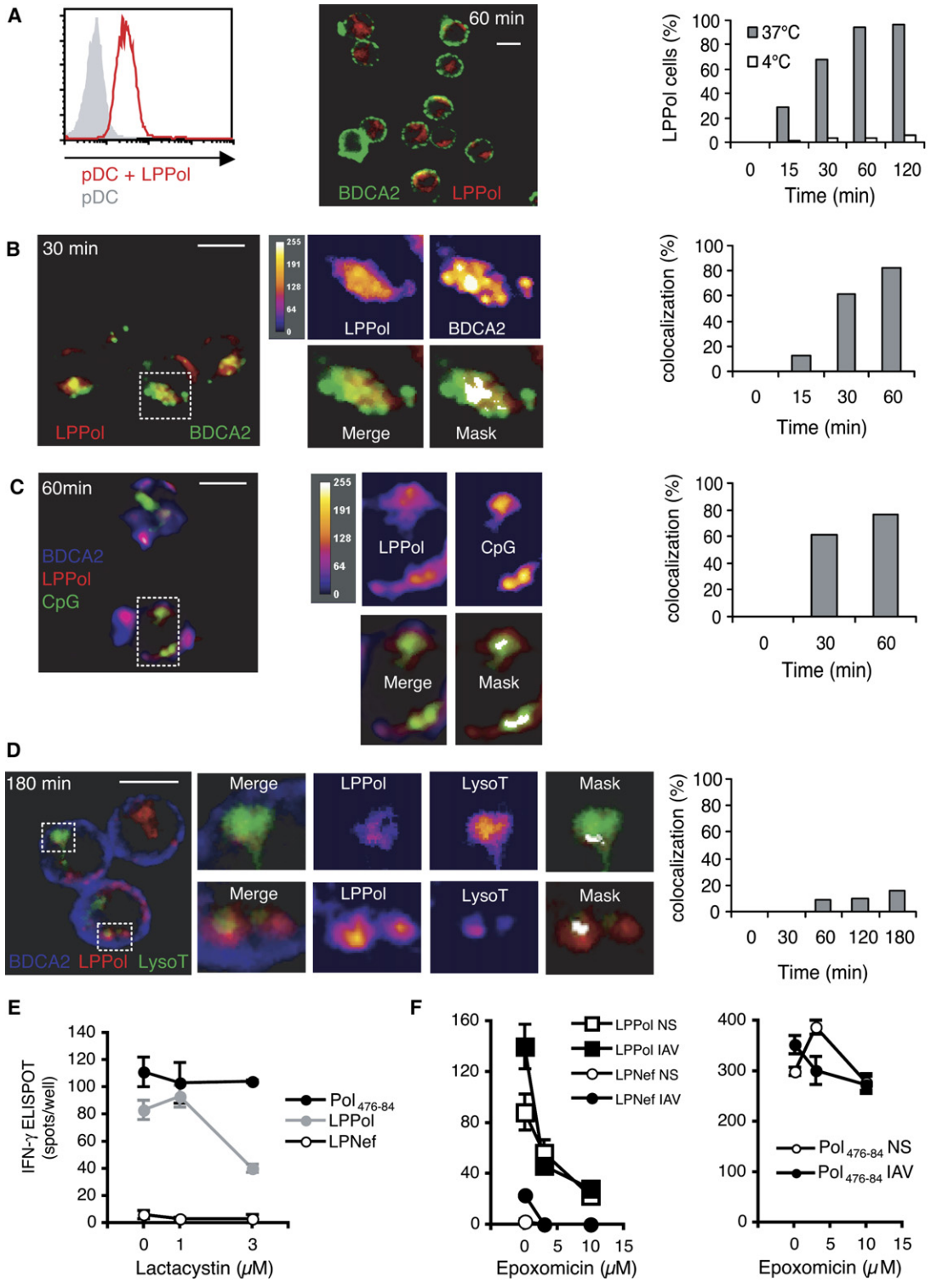

Figure 3. Uptake and Processing Pathway of the LPPol Lipopeptide

(A) Left, FACS histogram showing purified pDCs incubated (red) or not (shaded) with 1 μM LPPol-Rho for 1 hr. Middle: confocal microscopy showing the same pDCs, surface-labeled with anti-BDCA-2. Right: the percent of cells with $LPPol^+$ vesicles increases with time at 37°C and not at 4°C.

(B) LP-Pol-Rho colocalizes with FITC-anti-BDCA-2-labeled vesicles after incubation at 37°C for 30 min. Left: color merge; selected area is enlarged in the middle. Middle: single channel intensity false-color labeling. Bottom middle: the mask shows maximal overlapping pixels (colocalization coefficient > 0.8). Right: frequency of pDCs (n = 150) with colocalization increases with time.

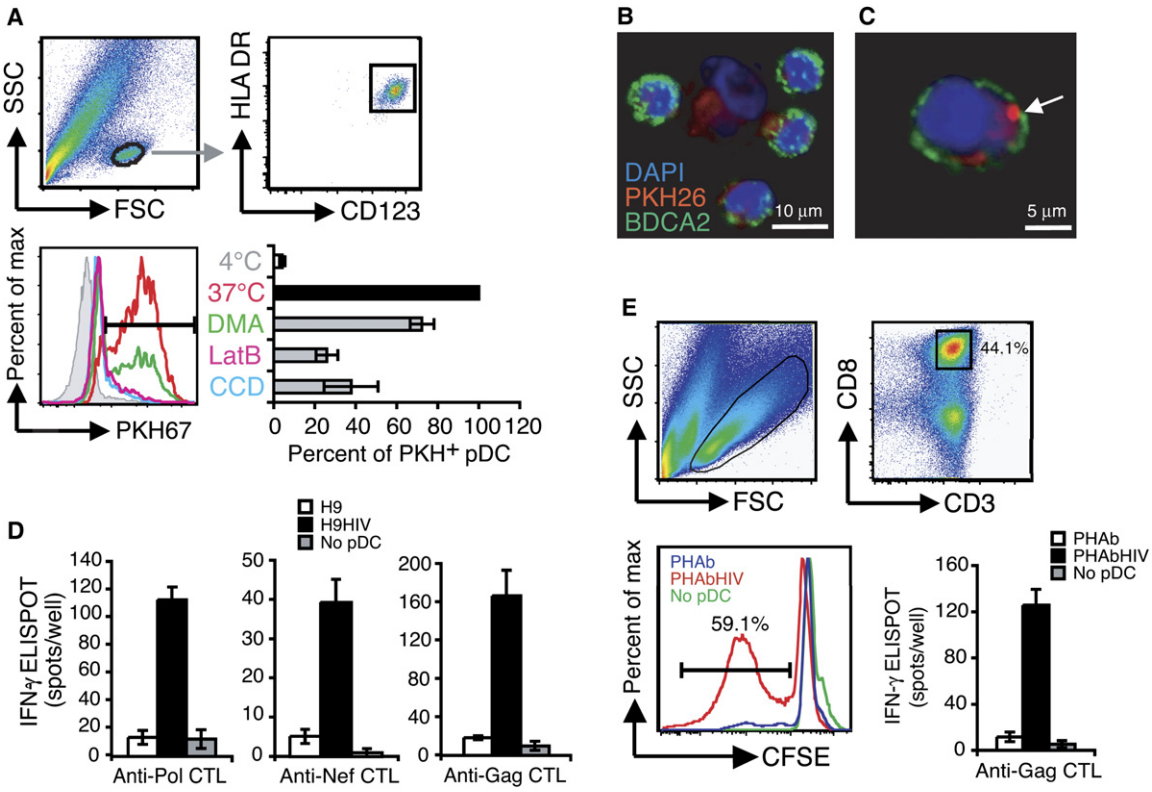

Figure 4. pDCs Capture Apoptotic Infected T Cells and Crosspresent HIV Antigens

(A) PDCs were treated or not with different endocytosis inhibitors, washed, and incubated with PKH67-labeled apoptotic H9HIV cells at 4°C or 37°C. Then, pDCs were washed and labeled with anti-HLA DR and anti-CD123 and analyzed by FACS. Results are represented as mean ± SEM of three independent experiments.

(B and C) pDCs engulf apoptotic fragment from infected T cells. Plasmacytoid DCs were incubated with PKH26-labeled apoptotic H9-HIV cells and analyzed by confocal microscopy after anti-BDCA2 and DAPI labeling.

(D) Plasmacytoid DCs present HIV antigens from apoptotic infected T cells to $CD8^+$ T cell lines. H9HIV-loaded pDCs were stimulated with R-837 or CpG, cultured with AZT, and used as APCs with Gag-, Nef-, or Pol-specific T cells in an IFN-γ ELISPOT assay. As controls, H9HIV cells were added without pDCs (no pDC).

(E) Plasmacytoid DCs present HIV antigens from apoptotic, infected PBMC. PHA blasts were infected or not with HIV_{Lai}, treated with SQ and AMD, and incubated with pDCs in the presence of R-837. Then, loaded pDCs were incubated with CFSE-labeled PBMCs from an HIV^+ patient, in the presence of SQ and AMD. After 6 days, $CD3^+$ $CD8^+$ T cell proliferation was analyzed by FACS. Alternatively, loaded pDCs were tested with Gag-specific T cells in an ELISPOT assay as in (D). As a control, apoptotic HIV-infected PHA blasts were added without pDCs. All data are represented as mean ± SEM (n = 3 replicates) and representative of at least three independent experiments.

Presentation by pDCs often leads to other T cell responses, such as IL-10 secretion, and to tolerance (Tang and Bluestone, 2006). This may explain why crosspresentation by pDCs was not shown in other experimental settings (Schnurr et al., 2005). Indeed, human pDCs can induce $CD4^+$ or $CD8^+$ Treg cells in vitro after stimulation with CpG (Moseman et al., 2004), CD40L (Gilliet and Liu, 2002), or HSV-1 (Kawamura et al., 2006). In vivo, pDCs also induce alloantigen-specific Treg cells (Ochando et al., 2006). In addition, depletion of pDCs renders mice susceptible to asthma in response to inhaled OVA, which is presented by $CD11c^+$ DCs (van Rijt et al., 2005). In this

(C) LP-Pol-Rho colocalizes with DOTAP-CpG-FITC; pDCs were incubated first with the DOTAP-CpG-FITC (green) and washed, and then LPPol-Rho (red) was added for 60 min at 37°C. Afterwards, pDCs were washed and surface labeled at 4°C with Cy5-anti-BDCA-2 (blue). Left: color merge; selected area is enlarged in the middle. Middle: single channel intensity false-color labeling. Right: frequency of pDCs with colocalization.

(D) LPPol-Rho mostly does not colocalize with LysoTracker. Left: LPPol-Rho (red) and LysoTracker (green) were incubated at the same time, then stained with Cy5-anti-BDCA-2 (blue). Selected areas are enlarged in the middle (color merge, single channel intensity false-color labeling and colocalization mask). Right: frequency of pDCs with colocalization is low, starting only at 60 min. Data are represented as mean and representative of at least two experiments.

Scale bars represent 10 μm.

(E and F) Proteasome processing is required for LPPol crosspresentation. PDCs were treated with proteasome inhibitors (E) lactacystin or (F) epoxomycin 30 min before loading with LPPol as before and used in an IFN-γ ELISPOT assay. NS, nonstimulated. Data are represented as mean ± SEM (n = 3 replicates) and representative of at least four independent experiments.

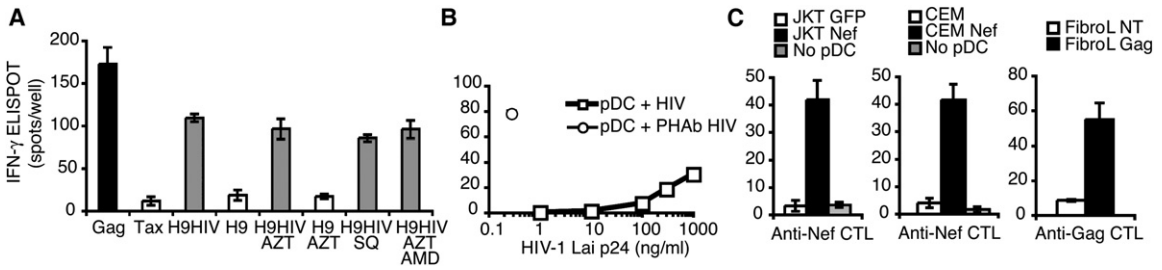

Figure 5. Direct Antigen Presentation after pDC Infection Does Not Account for the T Cell Responses Observed during HIV Antigen Crosspresentation by pDCs

(A) Plasmacytoid DCs were loaded with apoptotic H9HIV cells in the presence of IL-3 and of different antiretroviral drugs. Then, pDCs were used as APCs with HIV-1 Gag-specific $CD8^+$ T cells in an IFN-γ ELISPOT assay.

(B) Comparison between crosspresentation and direct presentation responses. pDCs were loaded for 12 hr with apoptotic primary PHAbHIV (that contained $HIV\text{-}1_{Lai}$ as measured in Figure S3) in the presence of AZT and AMD (dot) or incubated with growing amounts of $HIV\text{-}1_{Lai}$ without inhibitors (squares) and stimulated with CpG, then used as in (A).

(C) Crosspresentation of different HIV-1 protein-expressing cells by pDCs. Plasmacytoid DCs were loaded with apoptotic cells: either Jurkat or CEM cells expressing Nef or murine fibroblasts transiently transfected with *gag*; they were then used as in (B). Data are represented as mean ± SEM (n = 3 replicates) and are representative of two independent experiments in (A) and (C) and three in (B).

model, pDCs induce OVA-specific Treg cells (de Heer et al., 2004). In HIV infection, pDCs crosspresenting HIV antigens may thus participate in Treg cell induction, a phenomenon suggested to play a role in pathogenesis (Boasso et al., 2006).

Tolerance can be broken by IAV infection. Soluble, endotoxin-free OVA is a weak stimulator for $CD8^+$, $CD4^+$ T cell and antibody responses, but becomes strongly immunogenic when IAV is administered 2–3 days prior to OVA (Brimnes et al., 2003). Similarly, peptide-loaded pDCs prime $CD8^+$ T cells in vivo when they are stimulated by IAV, and not by CpG (Schlecht et al., 2004). Intercurrent IAV infection in mice sensitized intranasally to OVA either breaks immunological tolerance when it is administered simultaneously to OVA, or induces Th1-mediated protection when it is administered later (van Rijt et al., 2005). It will be of great interest to determine whether this is due to crosspriming by pDCs.

In summary, crosspresentation by pDCs has strong potential implications in physiology or pathogenesis. First, it may induce tolerance most of the time, but intercurring infection or other stimulations may break this tolerance and yield unwanted allergic or autoimmune responses. Second, the ability of pDCs to crosspresent antigens should be taken into account for vaccination or immune therapies, in order to trigger and stimulate the desired subsets of DCs, to induce either tolerance or full effector responses.

EXPERIMENTAL PROCEDURES

Peptides, Lipopeptides, Antibodies, and Virus

$HIV\text{-}1_{Lai}$ HLA-A2-restricted peptides $Pol_{476\text{-}484}$ (ILKEPVHGV), $Tax_{11\text{–}19}$ (LLFGYPVYV), and $Gag_{77\text{-}85}$ (SLYNTVATL) and HLA-A3-restricted $Nef_{73\text{-}82}$ (QVPLRMTYK) were from Neosystem (Strasbourg, France). LPPol (NH_2-K(Nε-Pam)PLTEEAELELAENREILKEPVHGV-COOH including $Pol_{461\text{-}484}$), LPPol-Rho (substitution of His_{482} by Nε-rhodamine-Lys), LPNef (NH_2-GVFPVTPQVPLRPMTYKAAVDLSHFLKEKGGL(Nε-Pam)-COOH, including $Nef_{66\text{-}97}$) (Andrieu et al., 2003), and the vaccinal lipopeptide preparation LPHIV1, from ANRS, were used at 1 μM of each lipopeptide. Sequences included in LPHIV1 were TT-NEF:K-GRQYIKANSKFIGITERGRFPVTPQVPLRPMTYK; TT-POL1:K-GRQYIKANSKFIGITERGRNPDIVIYQYMDDL; TT-RT:K-GRQYIKANSKFIGITERGRILKEPVHGV; TT-GAG: K-GRQYIKANSKFIGITERGRSLYNTVATL.

Monoclonal Abs directed against the following molecules were used: CD11c (pure, PE or APC), lineage-FITC, HLA DR-PerCP, CD3-APCCy7 (Becton Dickinson, BD), CD8-PerCP (Caltag), CD123-APC, BDCA-2 (pure, PE; Miltenyi Biotech), HLA-A2 (BB7.2), HLA-A3 (GAPA3), and MHC class I (W6/32, kind gifts from F. Connan). Goat anti-mouse Ig (GaMIg)-FITC and GaMIg-Cy5 were from Caltag.

A mouse-adapted strain of influenza A/Puerto Rico/8/34 virus (PR8) was grown in 10-day-old embryonated hen eggs. Allantoic fluid was harvested on day 2 and clarified by centrifugation at 1500 × g for 15 min. Aliquots were stored at −80°C. Titers were determined by standard plaque assay in the presence of 2 μg/ml TPCK-treated trypsin with Madin-Darby canine kidney cells.

Cell Purification, Culture, Transfection, and HLA Typing

HLA-typed buffy coats or cytapheresis residues were obtained from healthy donors within a convention with INSERM from French Blood Bank (EFS-Alsace) or from Transfusion Medicine Unit, Cochin Hospital, with informed consent from all subjects and according to ethical guidelines. PBMCs separated by Ficoll-Paque (Pharmacia) were first depleted of B cells by $CD19^+$ MACS microbeads. mDCs were selected from the $CD19^-$ fraction with BDCA-1 microbeads (Miltenyi). The mDC^- fraction was then further depleted for non-DCs with the DC-negative selection kit (Dynal), and finally pDCs were isolated by BDCA-4-positive selection kit (Miltenyi Biotech). In some cases, pDCs were selected from the $lineage^-$, mDC^- fraction by Diamond pDC isolation kit (Miltenyi). PDCs were routinely 94%–99.2% pure with a median of 96.5% and mDCs were 94% pure, as assessed by FACS Canto analysis (BD). Myeloid DCs and pDCs were maintained in complete medium (CM: RPMI 1640-glutamax containing 100 U/ml penicillin; 100 μg/ml streptomycin, 1% nonessential amino acids, 1 mM sodium pyruvate, 10 mM HEPES buffer, Life Technologies) + 10% heat inactivated autologous plasma. They were cultured in polypropylene tubes at 2.5×10^5/ml with either IL-3 (R&D Systems, 10 ng/ml), LPS (Sigma, 500 ng/ml), CpG A phosphorothioate (2216, 3 μM, Invitrogen), imiquimod (Invivogen, Toulouse, France, R-837, 10 μM), or IAV (1 PFU/cell).

HIV-specific $CD8^+$ primary T cell lines were generated with PBMCs from HLA-A2 or A3 HIV^+ individuals from cohort studies with the approval of Cochin Hospital's ethics committee. They were cultured

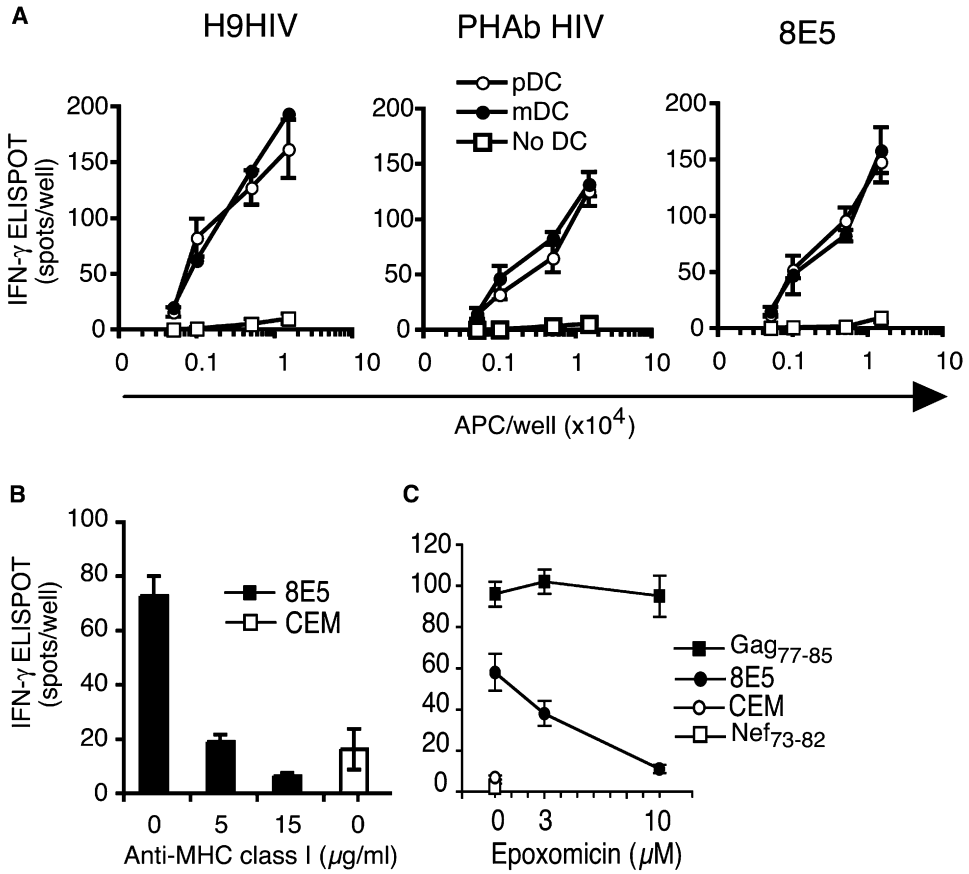

Figure 6. HIV Antigen Processing by pDCs

(A) mDCs and pDCs induce equivalent responses to T cell lines. Growing amounts of pDCs or mDCs were incubated with apoptotic H9HIV (left), HIV-PHA blasts (middle), or 8E5 cells (right) in the presence of CpG or LPS, respectively, and of SQ and AMD, and used as APCs with HIV-1 Gag-specific $CD8^+$ T cells in an IFN-γ ELISPOT assay. Alternatively, apoptotic cells were used without DCs in dose-response curves.

(B) Loaded pDCs were incubated with increasing concentrations of anti-MHC class I and used as APCs as in (A).

(C) pDCs were treated with epoxomycin before loading with apoptotic cells and used as APCs as in (A). Data are represented as mean ± SEM (n = 3 replicates) and are representative of two independent experiments in (B) and three in (A) and (C).

in CM + 5% human serum (Biowest, Nouaillé, France), stimulated with 1 μM $Pol_{476-484}$, Gag_{77-85} (HLA-A2-restricted), or Nef_{73-82} (HLA-A3-restricted) peptide, and maintained for 2–3 weeks at 0.7 × 10^6–1 × 10^6 cells /ml, adding IL-2 (10 U/ml, Roche) twice a week.

Uninfected or chronically infected H9 cells were cultured as before (Marañón et al., 2004). PHA blasts were prepared as follows: PBMCs isolated from a healthy donor (HLA $A2^-$ $A3^-$) were cultured at 0.5 × 10^6/ml with PHA (Sigma, 1 μg/ml) and 10 U/ml IL-2 for 2 days, then infected or not with 100 ng/ml p24 equivalent concentration of HIV_{Lai} for 12 hr.

8E5 cells are derived from CEM cells and contain a defective proviral HIV genome that produces no infectious virus. CEM clones stably expressing the HIV-1_{Lai} *nef* gene were generated by transduction of CEM cells with a murine retroviral vector (Schwartz et al., 1993). Jurkat cells expressing HIV-1 *nef* were obtained by transient transduction with a lentiviral vector as described (Sol-Foulon et al., 2004).

L cells were transfected via Fugen (Roche Diagnostics) with a plasmid prepared as follows. An HIV *gag* cDNA clone from the Lai isolate (accession #K02013) was digested with EcoRI and MunI and cloned in the EcoRI restriction site of pEGFP-N3 (Clontech, Mountain View, CA), so that the first 374 amino acids of Gag were expressed as a protein fused to eGFP.

HLA typing of different cells was performed by molecular biology methods in the HLA-typing facility of the Blood bank, EFS-Alsace, Strasbourg: H9 (HLA-A*01; B*15 -B*1501 most probable, equivalent to B62 serological typing), CEM and 8E5 (HLA-A*01, *31; B*08, *40 -*4001 most probable, equivalent to B60 serological typing), Jurkat (HLA-A*03; B*07, *35). HLA-A2 or -A3 expression was tested on PBMCs from healthy donors by mAb labeling and FACS.

Lipopeptide or Apoptotic Fragment Uptake

To follow lipopeptide acquisition by pDCs or mDCs, PBMCs were incubated with different concentration of LPPol-Rho in CM (37°C, 30 min). Cells were then extensively washed at 4°C in PBS, 5% autologous plasma, and 2 mM EDTA and surface labeled with anti-Lineage-FITC, anti-HLA DR-PerCP, and either anti-CD11c-APC or anti-CD123-APC, before analyzis by FACS. To study apoptotic fragment engulfment, H9 or H9HIV cells were labeled with PKH26 or 67 according to manufacturer instructions (Sigma), extensively washed, and UV-irradiated at 312 nm for 30 min. After 24 hr, apoptotic cells were treated with bicyclam AMD3100 (AMD, Sigma, 5 μg/ml) and incubated for 8 hr at 37°C at a ratio of 1:1 with pDCs preincubated for 30 min or not with LatB (1 μM), CCD (10 μM), or Dimethylamiloride (DMA, 50 μM; all from Calbiochem). Apoptotic cell fragment capture by pDCs was

analyzed by FACS according to size/granularity criteria and HLA DR and CD123 expression or by confocal microscopy.

Confocal Microscopy

Purified pDCs were incubated with 1 μM LPPol-Rho in CM at 37°C, extensively washed in PBS, 5% autologous plasma, and 2 mM EDTA and labeled with anti-BDCA-2 mAbs (30 min, 4°C), washed, and labeled with GaMIg-FITC (30 min, 4°C). To analyze the LPPol endosomal pathway in pDC, cells were first labeled with anti-BDCA-2 as described before, then incubated with 1 μM LPPol-Rho for different times at 37°C. Endosomal compartments were also labeled (30 min, 37°C) with FITC-CpG B (Invivogen, 1 μM) complexed with 1,2 dioleoyloxy-3-trimethylammonium-propane (DOTAP, Roche Diagnostic) (Honda et al., 2005), then cells were extensively washed and incubated with 1 μM LPPol-Rho at 37°C. Acidic organelles were visualized with 100 nM LysoTracker green DND 26 (Molecular Probes) during LPPol-Rho incubation. Cells were washed in PBS, fixed in 4% paraformaldehyde, and mounted (Fluorescent Mounting Medium, Dako Cytomation). Fluorescent images were acquired on an inverted Leica LSM TSC SP2 AOBS confocal microscope (63× oil-immersion objective, 1.4 NA plan apochromat). Multiple color images were acquired by scanning in sequential mode to avoid crossexcitation. Images were analyzed with ImageJ 1.37c and colocalization finder from ImageJ. Colocalization frequencies were quantified by counting cells (n = 150) respecting mask criteria (Pearson coefficient = 0.8), with a minimal threshold of fluorescence intensity for both fluorochromes and a ratio of 80%.

Crosspresentation

Antigen donor cells were washed, UV-irradiated (312 nm) for 20 min (45 min for fibroblasts), and incubated at 2×10^6 cells/ml in CM + 10% heat inactivated plasma for 8 hr. Maturation stimuli, AZT (Sigma; 200 nM), Saquinavir (SQ, Roche, 1 μM), or AMD were added as indicated. 50,000 pDCs or mDCs were cultured with 1 μM lipopeptide or with apoptotic cells at a ratio of 1:1 in CM + 10% heat inactivated autologous plasma for 12 hr. After loading, DCs were extensively washed and used (7,000–15,000/well) in a 12–16 hr IFN-γ ELISPOT assay (Fonteneau et al., 2003) with T cell lines as effectors (21,000–45,000/well). DCs from HLA-$A2^+$ donors were used in most experiments, but DCs from HLA-$A3^+$ donors were used when HLA-A3-restricted, Nef_{73-82}-specific T cell responses were tested. HLA typing of DCs and antigen donor cells and HLA restriction of the observed response are shown for each experiment in Table S1. In some experiments, $CD4^+$ T cells and $CD8^+$ T cells were depleted or enriched with anti-CD4 or -CD8 mAb-coated magnetic beads (Miltenyi). To inhibit proteasome activity, pDCs were treated 30 min before lipopeptide or apoptotic cell loading and during the whole period of antigen loading with epoxomycin (Alexis) or lactacystin (Calbiochem). All conditions were tested in triplicate.

$CD8^+$ T Lymphocyte Proliferation

$HIV\text{-}1_{Lai}$-infected or uninfected PHA blasts from a non-HLA-A2 healthy donor were washed and cultured for 24 hr in the presence of SQ and UV irradiated. HLA-$A2^+$ pDCs were loaded with apoptotic cells at a ratio of 1:1 in the presence of R-837, SQ, and AMD for 24 hr. They were then cultured in SQ and AMD with primary CFSE-labeled PBMCs (1 μM; Molecular Probes) from an HLA-$A2^+$ HIV^+ patient, at a pDC:PBMC ratio of 1:5. Residual APCs were removed from PBMCs by adhesion. 5 to 10 U/ml of IL2 (Roche) was added at days 1 and 3 to substitute for the lack of helper cells. After 6 days, cells were labeled for CD3 and CD8 and analyzed by FACS.

Direct Presentation

Viral amounts contained in apoptotic cells were evaluated by p24 ELISA (Innotest HIV Antigen mAb kit, Innogenetics). Different amounts of free virus were incubated with pDCs in an ELISPOT assay. 5×10^4 pDCs were incubated with H9 or H9HIV or equivalent amount of free $HIV\text{-}1_{Lai}$ virus and stimulated or not with IAV for 12 hr. Supernatants were collected after 48 or 72 hr and incubated with 10^5 CEM cells to amplify infectious virus. After 20 days, the virus contained in the supernatant was evaluated by p24 ELISA.

Supplemental Data

Three figures and one table are available at http://www.immunity.com/cgi/content/full/27/3/481/DC1/.

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